Degradation of Inorganic Polyphosphate in Mutants of \textit{Aerobacter aerogenes}

F. M. HAROLD AND RUTH L. HAROLD

\textit{Division of Research and Laboratories, National Jewish Hospital, and Department of Microbiology, University of Colorado School of Medicine, Denver, Colorado}

Received for publication 21 December 1964

\textbf{Abstract}

Harold, F. M. (National Jewish Hospital, Denver, Colo.), and Ruth L. Harold. Degradation of inorganic polyphosphate in mutants of \textit{Aerobacter aerogenes}. J. Bacteriol. 88:1262-1270. 1965.—Extracts of \textit{Aerobacter aerogenes} contained two enzymes capable of degrading polyphosphate, polyphosphatase and polyphosphate kinase. By use of a suicide technique, a mutant (Pn-4) blocked in polyphosphate degradation was isolated; this mutant was found to lack polyphosphatase. The results indicate that polyphosphatase mediates the main pathway of polyphosphate degradation, and, therefore, that polyphosphate does not serve as a microbial phosphagen. A second mutant (Pn-3) exhibited transient accumulation of polyphosphate when cells were transferred to fresh growth medium. This strain was constitutive for elevated levels of polyphosphate kinase, polyphosphatase, and alkaline phosphatase; the transient accumulation of polyphosphate may be due to the shifting ratios of the biosynthetic and degradative enzymes during growth. These mutants were employed in studies on the competitive relationship between polyphosphate and nucleic acids. It was concluded that nucleic acid synthesis inhibits polyphosphate synthesis and also stimulates polyphosphate degradation.

The level of inorganic polyphosphate in microbial cells is a function of their nutritional state. As a general rule, the polyphosphate content is low in growing cells, rises strikingly under certain conditions of nutritional imbalance, and falls again if rapid growth resumes (Kuhl, 1960; Harold, 1963a). Exploration of the regulatory mechanisms involved is of intrinsic interest, and may also shed light on the biological functions of polyphosphate.

In \textit{Aerobacter aerogenes}, as in many other microbial species, polyphosphate accumulation is induced by two distinct nutritional conditions (Smith, Wilkinson, and Duguid, 1954). (i) Polyphosphate slowly accumulates in cells whose growth has ceased due to exhaustion of an essential nutrient, such as sulfur (Harold, 1963a). (ii) Rapid and extensive polyphosphate accumulation takes place upon addition of orthophosphate (P\textsubscript{1}) to a suspension of cells previously subjected to phosphate starvation. We have designated the latter phenomenon the “polyphosphate overplus” (Harold, 1964). The difference between the two patterns was found to be due to nutritional control of the biosynthetic enzyme, polyphosphate kinase. Synthesis of this enzyme is repressed as long as P\textsubscript{1} is available to the cells, but its specific activity increases considerably during phosphate starvation. The rapid accumulation of polyphosphate induced by addition of P\textsubscript{1} to the starved cells is presumably a consequence of the elevated level of polyphosphate kinase. Analysis of two mutants blocked in polyphosphate accumulation (Harold and Harold, 1963) supported this interpretation. Mutant Pn-1 accumulates polyphosphate upon sulfur starvation but does not exhibit the overplus phenomenon; this mutant contains low levels of polyphosphate kinase, but repression of enzyme synthesis is not released by phosphate starvation. Mutant Pn-2 lacks polyphosphate kinase altogether and fails to accumulate polyphosphate under any conditions (Harold, 1964). In general, the rate of polyphosphate synthesis appears to be proportional to the level of polyphosphate kinase.

The polyphosphate content must be a function of degradation as well as synthesis. Previous studies suggested that polyphosphate degradation in \textit{A. aerogenes}, and probably also in other microorganisms, is subject to metabolic control. Resting cells maintain large polyphosphate pools, but, if nucleic acid synthesis is initiated, the polyphosphate is rapidly mobilized and the phosphorus is quantitatively transferred to the nucleic acid fraction (Harold, 1963a). Further analysis of the relationship between polyphosphate and nucleic...
acid metabolism requires clarification of the chemical pathway of polyphosphate degradation. By now no fewer than four distinct enzymes, listed in Table 1, have been shown to degrade polyphosphate in extracts, but it is not known which of these mediates the reaction in living cells.

The purpose of the present report is to describe two novel mutants of *A. aerogenes*. Strain Pn-4 is deficient in polyphosphatase, and strain Pn-3 is constitutive for elevated levels of polyphosphatase, polyphosphate kinase, and alkaline phosphatase. The results indicate that polyphosphatase mediates the major route of polyphosphate degradation in this organism; the results also clarify some aspects of the relationship between nucleic acid and polyphosphate metabolism. A preliminary report concerning the constitutive mutant has been published (Harold and Harold, 1964).

**Materials and Methods**

Organisms. *A. aerogenes* A3(O) and mutants Pn-1 and Pn-2 were described previously (Harold, 1964). A uracil-requiring mutant of A3(O), designated O\(-\), served as the parent strain for the isolation of mutants Pn-3 and Pn-4 as outlined below. Both Pn-3 and Pn-4 retained the requirement for uracil. All growth experiments were done at 37°C in T\(\text{subscript}\)\(_{\text{aerogenes}}\) medium (Harold, 1963). This is a glucose-salts medium buffered with tris(hydroxymethyl)aminomethane (Tris) chloride; the subscript indicates the phosphate concentration in micrograms of phosphorus per milliliter.

Mutant Pn-3. Mutant Pn-3 was obtained by chance in the course of an unsuccessful attempt to apply a \(P^n\)-suicide method to the isolation of mutants blocked in polyphosphate degradation. Among the survivors from an experiment carried out with strain O\(-\) essentially as described previously (Harold and Harold, 1963), several clones were noted which contained metachromatic granules even during the exponential phase of growth; one of these clones was selected for detailed study.

Mutant Pn-4. A mutant blocked in the degradation of polyphosphate was isolated by a modification of the procedure of Lubin (1959, 1962), based on the following principle. When cells of *A. aerogenes* containing inorganic polyphosphate are subjected to phosphate starvation, nucleic acid synthesis depends upon mobilization of the polyphosphate as sole source of phosphorus. Under these conditions, a uracil-requiring strain given uracil-H\(^3\) should synthesize nucleic acids and thus incorporate tritium. Any mutants unable to degrade polyphosphate should be unable to synthesize nucleic acids owing to lack of phosphorus, and should thus incorporate much less tritium. Upon subsequent prolonged storage at low temperatures, the parental type would be inactivated and the mutants would be expected to survive.

The procedure was as follows. Cells of O\(-\), harvested from T\(\text{subscript}\)\(_a\) medium, were irradiated with ultraviolet light to inactive 99 to 99.9% (Harold and Harold, 1963), diluted with fresh medium, and grown overnight. The bacteria were then collected and grown for 18 hr in T\(\text{subscript}\)\(_a\) medium (plus uracil). The phosphate-starved bacteria were centrifuged and incubated for 1 hr in T\(\text{subscript}\)\(_a\) medium (no uracil) to induce maximal accumulation of polyphosphate. The cells were again centrifuged, washed free from P\(\text{a}\), and suspended in T\(\text{subscript}\)\(_a\) medium (no uracil) at a density of \(2 \times 10^9\) cells per milliliter. To 0.25 ml of this suspension was added 0.25 ml of uracil-H\(^3\) (125 \(\mu\)c; specific activity, 3,000 mc/m mole; New England Nuclear Corp., Boston, Mass.), and the suspension was incubated for 5 to 6 hr. The cells were then centrifuged, suspended in 25 ml of T\(\text{subscript}\)\(_a\) medium containing 10% glycerol, dispensed into tubes, and frozen at \(-70^\circ\)C. Periodically, a tube was thawed for determination of the viable count, which dropped to 0.01% in 18 days. The survivors were then plated out, and each clone was screened individually for its ability to degrade metachromatic granules: the cells were induced to accumulate polyphosphate as described above, washed free from P\(\text{a}\), and then incubated for 4 hr in T medium (plus uracil). Smears were prepared, stained for metachromatic granules (Laybourn, 1954), and examined under a microscope. A single clone was found to retain metachromatic granules under conditions of phosphate starvation, and proved to be blocked in polyphosphate degradation. This mutant was designated Pn-4.

When mutant Pn-4 was put through the uracil-H\(^3\) procedure used for its selection, we found, to

Table 1. Enzymes that degrade inorganic polyphosphate

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction</th>
<th>Reference*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyphosphatase</td>
<td>((P_i)_n \rightarrow n P_1)</td>
<td>a</td>
</tr>
<tr>
<td>Polyphosphate kinase</td>
<td>((P_i)<em>n + ADP \rightarrow ATP + (P_i)</em>{n-1})</td>
<td>b</td>
</tr>
<tr>
<td>Polyphosphate glucokinase</td>
<td>((P_i)<em>n + glucose \rightarrow glucose-6-phosphate + (P_i)</em>{n-1})</td>
<td>c</td>
</tr>
<tr>
<td>Polyphosphate-AMP-phosphotransferase</td>
<td>((P_i)<em>n + AMP \rightarrow ADP + (P_i)</em>{n-1})</td>
<td>d</td>
</tr>
</tbody>
</table>

* References: (a) Muhammed, Rodgers, and Hughes, 1959; (b) Kornberg, 1957; Hoffmann-Ostenhof and Slecha, 1958; (c) Szymona and Ostrowski, 1964; Dirheimer and Ebel, 1962; (d) Winder and Denueney, 1957; Dirheimer, 1964.
our dismay, that it was inactivated as rapidly as was the parent strain $O_{3-}$. It is, therefore, likely that the successful isolation of this mutant was a stroke of good fortune rather than the product of a rational selection procedure. Nonetheless, the principle employed may prove useful as a general basis for the selection of mutants blocked in the metabolism of reserve substances.

**Analytical methods.** Sampling of cultures and the fractionation of phosphorus compounds were described previously (Harold, 1964), as were the preparation of extracts and the assay of alkaline phosphatase (Harold, 1964).

**Polyphosphatase.** Each tube received 0.05 ml of enzyme, 0.05 ml of 2 M KCl, 0.1 ml of $5 \times 10^{-3}$ M MgCl$_2$, 0.05 ml of P$_{32}$-polyphosphate (1 mg of P per ml), and 0.10 ml of Tris buffer (0.1 M, pH 9.0). The tubes were incubated at 37°C for 20 min, and the reaction was then stopped by addition of 1 ml of 1 M perchloric acid (PCA) and 0.1 ml of bovine serum albumin solution (10 mg/ml). The precipitate was removed by centrifugation, and the P$_{32}$-P$_2$ released was extracted into isobutanol (Harold, 1964). Addition of PCA and protein precipitated any remaining P$_{32}$-polyphosphate substrate almost completely, while P$_{32}$-P$_2$ remained in solution. In a few experiments, polyphosphatase was, therefore, assayed by the conversion of P$_{32}$-polyphosphate to acid-soluble material.

**Polyphosphate kinase.** This enzyme was assayed in the forward direction by the use of radioactive acetyl phosphate (Muhammed, 1961; Harold, 1964), and in the reverse direction by the transfer of P$_{32}$ from polyphosphate to adenosine diphosphate (ADP). For assay of the enzyme by its polyphosphate-ADP-phosphotransferase activity, each tube received 0.05 ml of enzyme, 0.05 ml of P$_{32}$-polyphosphate (1 mg/ml of P), 0.10 ml of Tris-maleate buffer (each 0.15 M, pH 8), 0.05 ml of ADP (0.02 M), 0.02 ml of MgCl$_2$ (1 M), and water to 0.40 ml. The mixture was incubated for 20 min at 37°C, and the reaction was stopped by addition of 0.1 ml of bovine serum albumin (10 mg/ml) and 0.50 ml of 1 N PCA. The tubes were centrifuged, the precipitates were washed once with 1 ml of 0.5 N PCA, and the supernatant fractions were pooled. The nucleotides were adsorbed onto Norit A (0.25 ml of a 100 mg/ml suspension). The Norit was washed three times with 0.5 N PCA, suspended in 2 ml of water, and 1 ml was plated and counted. As before, the unit is defined as the amount of enzyme which transfers 0.01 μmole of phosphorus from polyphosphate to ADP in 10 min.

**Triphosphatase.** Each tube received 0.1 ml of enzyme, 0.1 ml of triphosphatase (1 mg/ml of P), 0.1 ml of MgCl$_2$ (5 × 10$^{-3}$ M), and 0.2 ml of Tris buffer (0.1 M, pH 7.5). The mixture was incubated for 20 min at 37°C; the reaction was stopped by addition of 1 ml of 0.5 N PCA, and the P$_2$ released was measured. Since crude extracts and some ammonium sulfate fractions released P$_2$, even in the absence of triphosphatase, control tubes without the substrate served as blanks. A unit of enzyme is defined by the liberation of 0.01 μmole of P$_2$ in 10 min.

**Purification of polyphosphatase.** Polyphosphatase was separated from alkaline phosphatase and triphosphatase by a combination of ammonium sulfate fractionation and gel adsorption, but the specific activity was increased only fourfold.

Strain A3(O) was grown on Trypticase Soy Broth (BBL). An amount of 10 g (wet weight) was suspended in 50 ml of Tris-maleate buffer (each 0.05 M, pH 6), treated for 10 min in a Raytheon 10-ka sonic oscillator, and centrifuged at 25,000 X g in the cold. To the supernatant liquid were added 15 ml of streptomycin sulfate (20 mg/ml) to precipitate the bulk of the nucleic acids without loss of enzyme. The precipitate was removed, and 10 g of solid ammonium sulfate were added to the supernatant fraction; the inactive precipitate was discarded. Addition of an additional 10 g of ammonium sulfate precipitated the bulk of the polyphosphatase, leaving over half the alkaline phosphatase and all the triphosphatase in solution.

The precipitate was dissolved in 20 ml of 0.05 M Tris-maleate buffer (pH 6) and dialyzed overnight against 0.005 M buffer containing 10$^{-3}$ M ethylene-diaminetetraacetate (EDTA). Calcium phosphate gel (Colowick, 1955) was then added fractionally to adsorb the polyphosphatase, leaving alkaline phosphatase in solution. The gel was washed with 0.05 M Tris-maleate buffer (pH 6) and dialyzed overnight against 0.05 M buffer (pH 6)–10$^{-3}$ M EDTA. The overall recovery was 20 to 30%.

**RESULTS**

**Polyphosphate degradation in cell-free extracts.** Extracts of *A. aerogenes* A3(O) and $O_{3-}$ were found to contain two enzymes, polyphosphatase and polyphosphate kinase, capable of degrading polyphosphate of high molecular weight.

**Polyphosphatase.** Degradation of P$_{32}$-polyphosphate to P$_{32}$-P$_2$ by crude extracts was described previously (Harold, 1964). Partially purified polyphosphatase required both Mg$^{++}$ and high concentrations of K$^+$ (see Materials and Methods) and had a broad pH optimum between pH 8 and 9. The enzyme was inhibited by phosphate: under our standard assay conditions, 0.05 M P$_2$, inhibited activity by over 90%, whereas 0.01 M P$_2$ had little effect. Nucleotides and a number of sugar phosphates (2 × 10$^{-3}$ M) did not inhibit polyphosphatase.

By the action of polyphosphatase, the P$_{32}$-polypolyphosphate was progressively converted into acid-soluble material, which was not precipitated when excess protein and PCA were added to the assay tubes. All the P$_{32}$ thus released was identified as P$_{32}$-P$_2$, and there was no evidence for the accumulation of polyphosphate intermediates small enough to escape precipitation.
Polyphosphate kinase. Kornberg (1957) demonstrated that polyphosphate kinase of Escherichia coli catalyzes the formation of adenosine triphosphate (ATP) from polyphosphate and ADP. This route of polyphosphate degradation is also found in A. aerogenes. Incubation of crude extracts of strain A3(O) with P32-polyphosphate and ADP resulted in the formation of a product that was adsorbed by Norit A. Other mono- and diphosphonucleosides could not substitute for ADP. The products of a large incubation mixture were eluted from the Norit with acetone-NH3 (Tsuboi and Price, 1959); carrier ATP, ADP, and adenosine monophosphate (AMP) were added, and the nucleotides were separated by ion-exchange chromatography (Smith and Khorana, 1958). About two-thirds of the P32 was eluted with the ATP peak, which was identified by the ratio of acid-labile to total phosphorus (0.65); the remainder of the P32 appeared to be ADP, but its identity has not been conclusively established.

Evidence for the identification of the polyphosphate-ADP-phosphotransferase with polyphosphate kinase was provided by the pattern of enzyme activity in various mutants. As shown in Table 2, transferase activity was absent in mutant Pn-2 (known to lack polyphosphate kinase); it was increased in A3(O) subjected to phosphate starvation, and was constitutively elevated in mutant Pn-3 (see below).

Other enzymes. Crude extracts hydrolyzed tri- polyphosphate to P1. Tripolyphosphatase was distinct from the polyphosphatase discussed above, and was readily separated from it by ammonium sulfate fractionation. It did not require KCl and did not hydrolyze P32-polyphosphate. Attempts to detect polyphosphate glucokinase and polyphosphate-AMP-phosphotransferase (Table 1) were unsuccessful.

Mutant deficient in polyphosphatase. Evidence concerning the pathway of polyphosphate degradation in vivo became available through the isolation of a mutant genetically blocked in polyphosphate degradation. Cells of strains O4 - and Pn-4 were harvested from an overnight culture in T200 medium plus uracil, washed, and incubated for 3 hr in T10 medium plus uracil. P1 was then added (100 μg of P per ml), and incubation was continued for 1 hr. The cells, which had accumulated large amounts of polyphosphate, were collected, washed, and suspended in T10 medium plus uracil. As shown in Fig. 1, polyphosphate in strain O4 - was rapidly degraded, and nucleic acid synthesis took place at its expense. By contrast, in strain Pn-4, polyphosphate degradation was very slow and little nucleic acid was synthesized.

Cell-free extracts of strain Pn-4 contained no detectable polyphosphatase. Polyphosphate kinase (assayed as polyphosphate-ADP-phosphotransferase), triphosphatase, and alkaline phosphatase were present in normal amounts, and increased in specific activity during phosphate starvation (Table 3). No evidence for the presence of an inhibitor of polyphosphatase could be obtained, nor did any polyphosphatase activity appear upon fractionation of Pn-4 extracts. It therefore appears that the block in polyphosphate degradation is due to the absence of polyphosphatase.

Competitive relationship between metabolism of polyphosphate and nucleic acids. Because of the block in polyphosphate degradation, mutant Pn-4 might be expected to accumulate polyphosphate even during the exponential phase of growth. This did not prove to be the case: growing cells of Pn-4, like those of the parent strain O4 -, were devoid of polyphosphate. In both strains, accumulation was induced only by deprivation of sulfur or uracil, or by the overplus procedure.

The explanation for the failure of growing cells to accumulate polyphosphate became apparent when nucleic acid synthesis was dissociated from growth by means of chloramphenicol (Harold, 1963a). Cells of strains O4 - and Pn-4

### Table 2. Polyphosphate-ADP-phosphotransferase activity in wild-type and mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Stationary phase</th>
<th>Phosphate-starved</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3(O)</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>Pn-2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pn-3</td>
<td>12</td>
<td>21</td>
</tr>
</tbody>
</table>

* Cells from overnight cultures on T200 medium were grown for 4 hr in T200 or T10 medium. For assay procedure, see Materials and Methods.
were subjected to sulfur starvation in T250 medium plus uracil, to induce polyphosphate accumulation in both. After 3.5 hr, each culture was divided; one half was incubated further, and chloramphenicol (10 \( \mu g/ml \)) and sulfate were added to the other half to stimulate nucleic acid synthesis. (It had been found earlier that a burst of amino acid-dependent nucleic acid synthesis occurs under these conditions.) In strain \( O^- \), as in A5(O) (Harold, 1963a), polyphosphate was rapidly degraded when nucleic acid synthesis was initiated; in strain Pn-4, polyphosphate accumulation ceased but its degradation was much slower (Fig. 2). Nucleic acid synthesis (omitted from Fig. 2) proceeded at the same rate in both strains. Since polyphosphate degradation in strain Pn-4 is blocked, the cessation of accumulation must be due to inhibition of polyphosphate synthesis by concurrent nucleic acid synthesis.

The inhibition of polyphosphate synthesis by concurrent nucleic acid synthesis was much less pronounced in phosphate-starved cells: upon addition of P, both polyphosphate and nucleic acids accumulated (Harold, 1964). This is presumably related to the high level of polyphosphate kinase produced by phosphate starvation. However, even in starved organisms, polyphosphate accumulation was both more rapid and more extensive if nucleic acid synthesis was prevented (e.g., by the absence of uracil).

The rate of polyphosphate degradation was also a function of concurrent nucleic acid synthesis. Cells of strain \( O^- \) were made to accumulate polyphosphate by the overplus procedure, washed, and resuspended in T2 medium. As shown in Fig. 3, the polyphosphate was largely retained during incubation in T2 medium, both in the presence and absence of glucose. However, when both glucose and uracil were provided, the polyphosphate was rapidly degraded and served as sole source of phosphorus for nucleic acid synthesis. These findings show that retention of the polyphosphate pool cannot be due to continuous degradation and resynthesis of polyphosphate, since an exogenous energy source is known from previous work to be required for polyphosphate synthesis. It would appear, rather, that polyphosphatase activity is inhibited in resting cells and is strongly stimulated by concurrent nucleic acid synthesis.

**Table 3. Enzymes of polyphosphate metabolism in mutants \( O^- \) and Pn-4**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Physiological state</th>
<th>Polyphosphate kinase</th>
<th>Alkaline phosphatase</th>
<th>Triphosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>( O^- )</td>
<td>Stationary phase</td>
<td>18</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Phosphate-starved</td>
<td>48</td>
<td>20</td>
<td>95</td>
</tr>
<tr>
<td>Pn-4</td>
<td>Stationary phase</td>
<td>0</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Phosphate-starved</td>
<td>0</td>
<td>25</td>
<td>94</td>
</tr>
</tbody>
</table>

* Cells were harvested after 4 hr of growth in T250 or T2 medium. Triphosphatase was measured in cells grown on Trypticase Soy Broth. Enzymes were assayed as described in Materials and Methods; polyphosphate kinase was measured by polyphosphate-ADP-phosphotransferase activity.

**Fig. 2. Effect of the resumption of nucleic acid synthesis on the polyphosphate level in strains \( O^- \) and Pn-4.** Cells of both strains were suspended in T2 medium (plus uracil, no sulfate) at 0 hr. At 3.5 hr (arrow), each suspension was divided; one part received no additions; the other, sulfate plus 10 \( \mu g/ml \) of chloramphenicol (CMP).
and Ojzymes polyphosphatase. Activity of the phosphatase, assayed in several times, the hr. (A) Significantly decreased during deprivation whereas that by the conditions of overplus. Cells made were harvested and washed, and suspended in T₅b medium (no glucose, no uracil). The suspension was divided, and additions were made as shown.

Phosphate occurred in strain Pn-3. Polyphosphate accumulation in strain Pn-3 could also be induced by the conditions effective with O₁₀⁻, i.e., nutrient deprivation and the overplus procedure.

The activities of polyphosphate kinase, polyphosphatase, and alkaline phosphatase were assayed in samples of the same culture. At all times, the specific activities of these three enzymes were considerably higher in Pn-3 than in the parent strain. The absolute values changed significantly during growth (Fig. 4B): the specific activity of polyphosphate kinase dropped, whereas that of polyphosphatase and alkaline phosphatase (not shown in the graph) increased. It thus appears that mutant Pn-3 is partially constitutive for elevated levels of all three enzymes. It is evident, however, that synthesis of polyphosphate kinase is still subject to repression by exogenous P₁₀⁻, whereas polyphosphatase and alkaline phosphatase accumulate without restraint. Synthesis of the three enzymes during growth is thus not coordinate, and the progressive shift in the ratio of polyphosphate kinase to polyphosphatase is presumably responsible for the transient accumulation of polyphosphate.

**DISCUSSION**

There are at least two potential routes for the degradation of polyphosphate in *A. aerogenes*, mediated by polyphosphatase and polyphosphate kinase. The finding that mutant Pn-4 is deficient in polyphosphatase and is simultaneously blocked in polyphosphate degradation provides clear evidence that the major route of degradation is the hydrolytic one. The sluggish degradation of polyphosphate in mutant Pn-4 (Fig. 1 and 2) points to the existence of an alternative pathway. Whether this route is catalyzed by polyphosphate kinase has not been determined, but in any event it appears to make at most a minor contribution to the overall rate of polyphosphate degradation. It follows that the energy stored in the phosphoanhydride linkages of the polyphosphate chain is largely dissipated when the material is mobilized, and therefore the widely accepted view (Kornberg, 1957; Hoffmann-Ostenhof, 1962) that polyphosphate serves as an energy reservoir.

![Fig. 3. Retention of the polyphosphate pool after overplus](image)

![Fig. 4. Transient polyphosphate accumulation during growth of mutant Pn-3](image)
Fig. 5. Polyphosphate cycle and its genetic control. \( Pn \) = polyphosphate. \( Pnase \) = polyphosphatase.

Analogous to the phosphagens of higher organisms is no longer tenable, at least for \( A. \) aerogenes. In other microorganisms, particularly the mycobacteria and corynebacteria, the phosphotransferases listed in Table 1 may permit partial conservation of the phosphate bond energy, but the significance of these enzymes in living bacteria has yet to be demonstrated.

The polyphosphate cycle in \( A. \) aerogenes as presently understood is shown in Fig. 5, together with a schematic outline of its genetic control. Since recombination analysis is not possible with this organism, our conclusions are based entirely on the metabolic patterns of mutant strains. Two strains are named to reflect mutations in structural genes, \( Pn-2 \) in the gene for polyphosphate kinase and \( Pn-4 \) in that for polyphosphatase. The synthesis of polyphosphate kinase, polyphosphatase, and alkaline phosphatase is controlled by at least one common regulator gene, because in mutant \( Pn-1 \) all three enzymes are permanently repressed and in mutant \( Pn-3 \) they are constitutively elevated. However, these three enzymes do not constitute an operon as defined by Ames and Martin (1964). Synthesis of the enzymes is not coordinate and, more conclusively, we have obtained partial revertants of mutant \( Pn-1 \) which have regained the capacity for derepression of alkaline phosphatase but not of polyphosphate kinase (unpublished data). The term "regulon" has been proposed (Maas and Clark, 1964) for a group of genes controlled by a common regulator gene but which do not fall within a single operon.

Both the synthesis and the degradation of polyphosphate are subject to metabolic control at two levels: that of enzyme synthesis and that of enzyme activity. The rate of polyphosphate synthesis depends upon the concentration of polyphosphate kinase (Harold, 1964). In addition, the activity of this enzyme is inhibited by concurrent nucleic acid synthesis (Fig. 2), perhaps as a result of competition for ATP, which is the metabolite common to both pathways. The pattern of polyphosphate metabolism in the constitutive mutant (Fig. 4) suggests that polyphosphate degradation likewise depends on the concentration of polyphosphatase. The rate at which the enzyme functions is markedly increased by concurrent nucleic acid synthesis (Fig. 3, see also Harold, 1963a). The nature of the coupling between polyphosphate degradation and nucleic acid synthesis remains elusive. A possible explanation is based on the finding that polyphosphatase is inhibited by \( P_1 \); nucleic acid synthesis would increase the demand for phosphorus and should deplete the intracellular \( P_1 \) pool, thereby relieving the inhibition of polyphosphatase. Unfortunately for this hypothesis, in exploratory experiments the induction of nucleic acid synthesis by chloramphenicol produced expansion of the intracellular \( P_1 \) pool, rather than the expected depletion. Thus the role of \( P_1 \) in the regulation of polyphosphatase activity is questionable.

The interactions among the four regulatory elements discussed above are sufficient to account in outline for the effects of nutritional conditions on the polyphosphate content of \( A. \) aerogenes. (i) In growing cells, the synthesis of nucleic acids both inhibits polyphosphate kinase and stimulates polyphosphate degradation, and thus the cells deposit little or no polyphosphate. If growth and nucleic acid synthesis cease owing to exhaustion of an essential nutrient, such as sulfur, polyphosphate degradation is inhibited. At the same time, the competition for ATP is relieved and polyphosphate then accumulates at a rate determined primarily by the level of polyphosphate kinase. (ii) Cells subjected to prior phosphate starvation contain elevated levels of kinase and are thus initially capable of rapid polyphosphate synthesis when \( P_1 \) is provided (overplus). With the resumption of growth, polyphosphate kinase is gradually repressed; at the same time, polyphosphate synthesis is progressively inhibited and its degradation is stimulated, by the resumption of nucleic acid synthesis. The polyphosphate initially accumulated is thus gradually mobilized. This is, of course, particularly striking if exogenous \( P_1 \) is removed after overplus. (iii) Finally, the transient accumulation of polyphosphate in growing cells of the constitutive mutant (Fig. 4) may be attributed to the shifting ratio of polyphosphate kinase to polyphosphatase.

The fairly complex regulatory mechanisms outlined above are consistent with the view that polyphosphate functions as a storage form of phosphate (Hughes and Muhammed, 1962;
Harold, 1963a). However, the concept of function implies that under some physiological conditions the presence of polyphosphate, or at least the capacity to produce it, should confer a physiological advantage. Numerous attempts to discover selective conditions which would favor the wild type of A. aerogenes A3(O) over mutants defective in polyphosphate metabolism have been unsuccessful, except for the observation that mutant Pn-2, which lacks polyphosphate kinase, is inactivated more rapidly upon prolonged storage than are the other strains. Thus, we must conclude that the function of polyphosphate is still unknown.

The accumulation of polyphosphate in many microbial species is associated with the formation of intracellular structures known to cytology as "volutin" or "metachromatic granules." The extensive evidence in support of the view that the polyphosphate is localized in these structures was reviewed by Wilkinson and Duguid (1960). Recently, however, Martinez (1963) concluded that the metachromatic granules of Spirillum volutans and of yeast are composed of poly-β-hydroxybutyrate and contain no polyphosphate. It is, therefore, noteworthy that throughout our studies with mutants defective in polyphosphate metabolism we have observed perfect correspondence between polyphosphate and the metachromatic granules—indeed, microscopy of stained preparations is part of the procedure for the screening of these mutants. There can thus be little doubt that, in A. aerogenes at least, the metachromatic granules do indeed contain polyphosphate.

ACKNOWLEDGMENTS

We thank Norma Layher and James Baarda for excellent technical assistance.

This investigation was supported by Public Health Service grant AI-03568 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED


SMITH, I. W., J. F. WILKINSON, AND J. P. DUGUID. 1954. Volutin production in Aerobacter aerogenes...


